Reduction in Glycated Hemoglobin and Daily Insulin Dose Alongside Circadian Clock Upregulation in Patients With Type 2 Diabetes Consuming a Three-Meal Diet: A Randomized Clinical Trial

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OBJECTIVE

In type 2 diabetes, insulin resistance and progressive β-cell failure require treatment with high insulin doses, leading to weight gain. Our aim was to study whether a three-meal diet (3Mdiet) with a carbohydrate-rich breakfast may upregulate clock gene expression and, as a result, allow dose reduction of insulin, leading to weight loss and better glycemic control compared with an isocaloric six-meal diet (6Mdiet).

RESEARCH DESIGN AND METHODS

Twenty-eight volunteers with diabetes (BMI 32.4 ± 5.2 kg/m^2 and HbA_{1c} 8.1 ± 1.1% [64.5 ± 11.9 mmol/mol]) were randomly assigned to 3Mdiet or 6Mdiet. Body weight, glycemic control, continuous glucose monitoring (CGM), appetite, and clock gene expression were assessed at baseline, after 2 weeks, and after 12 weeks.

RESULTS

3Mdiet, but not 6Mdiet, led to a significant weight loss (−5.4 ± 0.9 kg) ($P < 0.01$) and decreased HbA_{1c} (−12 mmol/mol, −1.2%) ($P < 0.0001$) after 12 weeks. Fasting glucose and daily and nocturnal glucose levels were significantly lower on the 3Mdiet. CGM showed a significant decrease in the time spent in hyperglycemia only on the 3Mdiet. Total daily insulin dose was significantly reduced by 26 ± 7 units only on the 3Mdiet. There was a significant decrease in the hunger and cravings only in the 3Mdiet group. Clock genes exhibited oscillation, increased expression, and higher amplitude on the 3Mdiet compared with the 6Mdiet.

CONCLUSIONS

A 3Mdiet, in contrast to an isocaloric 6Mdiet, leads to weight loss, significant reduction in HbA_{1c}, appetite, and overall glycemia, with a decrease in daily insulin. Upregulation of clock genes seen in this diet intervention could contribute to the improved glucose metabolism.

Diet intervention is a pivotal component of the medical management of diabetes (1). Treatment of insulin-resistant patients with type 2 diabetes with progressive β-cell failure usually starts with a diet intervention consisting of five or six small meals per day, with calories and carbohydrates uniformly distributed throughout the day (2–4)

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including a night snack before bedtime to avoid nocturnal hypoglycemia (5). Dietary intervention is usually accompanied by sequential addition of several antihyperglycemic agents, including glucagon-like peptide 1 (GLP-1) analogs and sodium–glucose cotransporter 2 (SGLT2) inhibitors (6). Despite this medical treatment, many patients require insulin therapy, which is gradually augmented according to the glucose target-driven strategy (7). However, this progressive increase in insulin dose often leads to weight gain (8), which may increase insulin resistance, leading to a vicious cycle further increasing insulin doses, continued weight gain, decreased likelihood of achieving glycemic targets, a high risk for diabetes complications and increased insulin dose-dependent cardiovascular risk and mortality (2). It is, therefore, important to prevent the weight gain when insulin treatment is required.

Although eating frequent small meals is advised as a means for weight loss and glycemic control (3,4,9), studies endorsing this practice are lacking. In fact, this meal distribution, especially the snacks consumed later in the day, has been associated with increased risk for obesity and type 2 diabetes with higher overall glycemia and glycated hemoglobin (HbA_{1c}) (10–12). Therefore, it is of utmost importance to schedule a more adequate meal frequency and optimal daily caloric and carbohydrate distribution to achieve weight loss and better glycemic control, allowing the reduction of insulin dose requirements.

Most of the metabolic processes involved in glycemic control (i.e., β-cell function, muscular glucose uptake, and hepatic glucose production) exhibit diurnal variations, which are controlled by the endogenous circadian clock (13). The circadian clock is found in the hypothalamic suprachiasmatic nucleus (SCN) and is synchronized by light. The molecular clock consists of self-sustained transcriptional-translational feedback loops (14). The transcriptional activators CLOCK and BMAL1 act as positive elements in the feedback loop. The CLOCK–BMAL1 complex drives transcription of the genes encoding the periods (PERs) and cryptochromes (CRYs) and the transcription factors REV-ERBα and RORα, thus maintaining the circadian (~24 h) oscillation. Similar clocks are found in peripheral tissues, such as muscle, liver, β-cells, α-cells, adipose tissue, and white blood cells (WBCs), and are controlled by the SCN clock and food availability (15–17). As human metabolism is optimized for food intake in the light period, while the dark period is optimal for fasting and sleeping (13,18,19), glycemic control is different throughout the day, showing maximal glucose elevation after identical foods consumed in the afternoon and evening compared with the morning in healthy individuals and those with type 2 diabetes (20–23).

In recent years, emerging evidence shows the influence of meal timing on the circadian clock and, as a result, on health and diseases. Several recent reports suggest metabolic disadvantages when high-calorie, high-carbohydrate foods are consumed during the evening hours. In contrast, when this eating pattern is shifted into morning hours, increased insulin sensitivity and lowered overall glycemia in obese and people with prediabetes or diabetes was observed (21–27). Studies in both rodents and humans have shown that increased meal frequency, with macronutrients evenly distributed across the day including at hours designed for sleep, result in disrupted rhythms or dampened circadian oscillations, promoting weight gain, increased lipid synthesis, fatty liver, and hyperglycemia (28,29). In contrast, reducing meal frequency to only two or three daily meals and shifting calories and especially carbohydrate intake to earlier hours of the day facilitates weight loss, improves glucose excursions, and reduces hunger and cravings in obese (22–24) and in patients with type 2 diabetes treated with oral antidiabetic agents (21,25,26). Moreover, recently, in an acute study, we showed that compared with breakfast skipping, high-energy breakfast rich in carbohydrates led to a significant upregulation of clock gene (*BMAL1*, *PER1*, *PER2*, *CRY1*, and *RORα*) expression in WBCs, which was associated with reduction of overall glycemia in healthy individuals and those with type 2 diabetes (30). These findings may suggest that enhanced clock gene expression, driven by a high-energy and carbohydrate intake at breakfast, may be the underlying mechanism for the improved overall glycemia.

We hypothesized that in patients with type 2 diabetes treated with insulin, a diet intervention consisting of three meals (3Mdiet), with high-energy and carbohydrate intake at breakfast and low-energy and carbohydrate intake at dinner, would upregulate clock gene oscillation, leading to a more effective weight loss, appetite, and glycemic control, allowing the reduction of the total daily insulin dose (TDID). We therefore compared the 3Mdiet with a pattern of six meals daily (6Mdiet), with calories and carbohydrates evenly distributed throughout the day. The dietary intervention was for 12 weeks, and body weight, glycemic control, continuous glucose monitoring (CGM), appetite, and clock gene expression were assessed.

RESEARCH DESIGN AND METHODS

Study Population

The study population initially included 44 individuals (19 men and 25 women), aged ≥25 years old with type 2 diabetes for ≥5 years, and treated with insulin for ≥1 year with TDID >25 units for at least 3 months prior to the study initiation. The study population had glycated hemoglobin (HbA_{1c}) ≥6.5% (≥47.5 mmol/mol) and BMI ≤45 kg/m^2 (Table 1). Participants with a normal sleeping schedule were included (i.e., sleep from ~2300 to ~0600). Night or rotating shift workers or those who crossed more than two time zones during the 2-week period prior to the study, those with insomnia for more than 3 nights per week, or those diagnosed with obstructive sleep apnea were also excluded. Subjects were sedentary at baseline and were asked to maintain their usual physical activity levels and report any change in their activity level. Participants were recruited at the Diabetes Unit, Wolfson Medical Center in Holon, Israel. The study was approved and monitored by the Institutional Review Board Helsinki Ethics Committee at Wolfson Medical Center. Each participant provided written informed consent. Recruiting period was between November 2016 and July 2017. Last visit of the study was October 2017. The study was registered on Clincaltrials.gov (NCT02709915).

Study Design

This was a randomized, controlled, parallel trial conducted over 15 weeks: 3 weeks of screening, recruitment, and baseline assessments and 12 weeks of diet intervention. Participants were

Table 1—Clinical characteristics at baseline

	All participants	3Mdiet ($N = 14$)	6Mdiet ($N = 14$)	P value
Male sex, number (%)	17 (61)	7 (50)	10 (71)	
Age (years)	68.8 ± 7	68 ± 8.6	69.5 ± 5.6	0.62
Duration of diabetes (years)	19 ± 7.7	18 ± 6.9	21 ± 8.4	0.12
Anthropometric measures				
Weight (kg)	91.4 ± 19.2	91.7 ± 18	91.1 ± 21	0.94
BMI (kg/m^2)	32.4 ± 5.2	32.1 ± 5	32.6 ± 5	0.79
Male WC (cm)	117.7 ± 14	116.4 ± 17	118.6 ± 12	0.76
Female WC (cm)	103.5 ± 10.3	106 ± 11	99 ± 8	0.30
Systolic blood pressure (mmHg)	139 ± 18	139.4 ± 16.5	138 ± 20	0.82
Diastolic blood pressure (mmHg)	67 ± 10	68 ± 11.4	66.5 ± 9	0.68
Diabetes control				
Fasting glucose (mg/dL)	164 ± 27	165 ± 25	164 ± 30	0.91
Fasting glucose (mmol/L)	9.1 ± 1.5	9.1 ± 1.5	9.1 ± 1.6	0.91
HbA_{1c} (%)	8.1 ± 1.1	8.2 ± 1	8 ± 1.2	0.65
HbA_{1c} (mmol/mol)	64.5 ± 11.9	65.6 ± 10.9	63.5 ± 12.9	0.66
Insulin treatment				
Duration of insulin treatment (years)	7.1 ± 5.6	6.6 ± 6.3	7.6 ± 4.9	0.64
TDID (units)	66 ± 40	60 ± 27.6	71.3 ± 49	0.46
Long-acting insulin (units)	43 ± 26	36.9 ± 17.4	49.4 ± 31	0.21
Short-acting insulin (units)	22.5 ± 19	23.1 ± 15.9	22 ± 22.4	0.50

Data are mean ± SEM unless otherwise indicated. WC, waist circumference.

randomly assigned to either one of two dietary interventions using a flip of a coin by a person not involved in the study: 3Mdiet or six 6Mdiet with the same total caloric intake (Fig. 1). Participants and clinicians were blinded to the randomization. Participants wore a CGM before, at the beginning, and at the end of the diet. A nutritionist assessed participants' adherence to the diet every 2 weeks. During the intervention, the participants had a biweekly appointment with a physician for the titration of the insulin dose according to the Treat-to-Target Trial algorithm (7). The primary end point was the change of TDID, while secondary end points included a change in clock gene expression in WBCs, body weight, glycemic control, and appetite and cravings scores.

Diet Intervention

Participants did not follow any specific diet or meal timing before the trial. Before the trial, participants reported eating multiple meals during the day, including snacking after dinnertime. The 3Mdiet and 6Mdiet were isocaloric and calculated by subtracting 500 kcal from the individual calculated Harris Benedict equation (1,500 ± 300 kcal) (Fig. 1). Both diets are shown in Supplementary Table 1. Both diets had the same macronutrient composition of fat, protein, and carbohydrates (35:25:40%, respectively), but with different meal timing, frequency, and caloric and carbohydrate distribution over the day (Supplementary Table 1). The 3Mdiet consisted of a large breakfast of 700 kcal, a medium-sized lunch of 600 kcal, and a small dinner of 200 kcal (22,31,32), whereas the 6Mdiet consisted of six meals (breakfast, lunch, dinner, and three snacks) with relatively uniform daily caloric distribution in the meals plus 150 kcal in each one of the three snacks (Supplementary Table 1). All of the participants were asked to eat breakfast before 0930 h, lunch between 1200 and 1500 h, and dinner between 1800 and 2000 h, whereas the 6Mdiet participants had additional three snacks at 1100, 1700, and 2200 h.

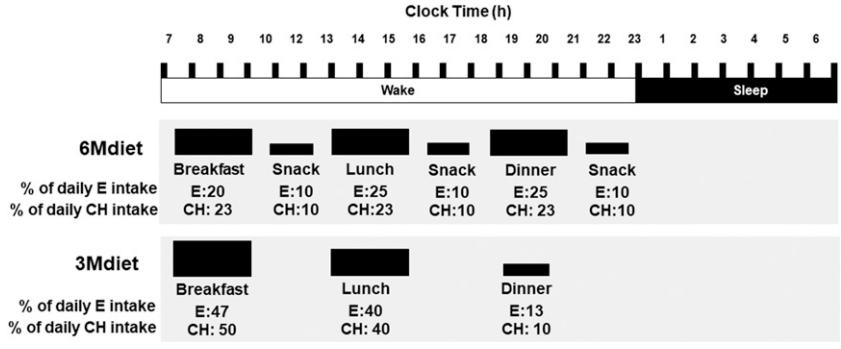

Figure 1—Mealtime and distribution of the 3Mdiet and 6Mdiet. CH, carbohydrates of daily carbohydrate intake; E, energy of the daily caloric intake.

Dietary Assessment and Compliance

The assigned diet was explained by a dietitian. The participants recorded their food intake and time. The dietitian provided personal counseling at the beginning of the diet and then every 2 weeks on the scheduled visits throughout the 3 months of the diet intervention. In addition, twice a week, the dietitian conducted a 10–15-min telephone conversation with the participants to confirm adherence with the assigned diet intervention. During the biweekly visits, the dietitian reviewed the diet records and provided counseling accordingly. Diet records were analyzed using Tzameret dietary analysis program (version 3) developed by the Israel Ministry of Health, and the compliance assessment was based on participant adherence to the diet and meal timing schedule. Noncompliance was defined as a deviation

of ≥10% from the recommended energy intake in a specific meal or mistake in the meal timing schedule or macronutrient distribution of the assigned diet (e.g., skipped breakfast or skipped snack). Participants with weekly noncompliance ≥42.9% (noncompliance of >3 days per week) were the threshold for withdrawal from the study.

Continuous Blood Glucose Monitoring

CGM was assessed by using Flash glucose-sensing technology: FreeStyle Libre (FSL-CGM Abbott Diabetes Care, Alameda, CA). The data from the CGM were downloaded by FreeStyle Libre software and were calculated separately for 24 h and the nocturnal segment (0000–0600 h).

Analysis of Gene Expression in WBCs

Blood for gene expression was collected in Tempus tubes (Applied Biosystems, Foster City, CA) and total RNA extracted according to the manufacturer's instructions. Total RNA was DNase I treated using RQ1 DNase (Promega, Madison, WI) for 2 h at 37°C, as was previously described (33). Two micrograms of DNase I–treated RNA were reverse-transcribed using Maloney murine leukemia virus reverse-transcriptase and random hexamers (Promega). One-twentieth of the reaction was then subjected to quantitative real-time PCR using primers spanning exon–exon boundaries (Supplementary Table 2) and the ABI Prism 7300 Sequence Detection System (Applied Biosystems). The fold change in target gene expression was calculated by the $2^{-\Delta\Delta Ct}$ relative quantification method using *Actin* as the housekeeping reference transcript (Applied Biosystems).

Biochemical and Hormonal Blood Analyses

Plasma glucose was immediately analyzed with hexokinase using a Cobas analyzer (Roche Diagnostics, Madison, WI). HbA_{1c} was determined by turbidimetric inhibition immunoassay for hemolyzed whole blood and analyzed with Cobas Integra 400 plus (Roche Diagnostics). Complete blood count was measured using the automated hematology system (XN-9000; Sysmex Corporation, Kobe, Japan).

Appetite and Craving Questionnaires

Appetite scores for hunger and desire for sweets were assessed using 100-mm visual analog scales. Participants rated their feelings of appetite (hunger and desire for sweets) by making a single vertical mark on each scale somewhere between the 0 and 100 mm extremes (e.g., with "not at all" and "very much/extremely" defining the extremes) to indicate hunger rate and desire for sweets at that time point. On the day of evaluation, appetite and desire for sweets were assessed once at fasting, three time points between breakfast and lunch, between lunch and dinner, and after dinner but before sleep. Food cravings were also assessed using the Food Craving Inventory, a 28-item validated questionnaire designed to measure the frequency of overall daily food cravings as well as craving sensation for specific types of foods (sweets, carbohydrates and starchy food, fast food, and high-fat food) (34).

Sample Size and Power Analysis

A sample size of $n = 28$ (14 in each group) was required for a pairwise comparison with an overall power of 95% to detect a true, between-group difference of 40 ± 25% in TDID, the primary end point at the end of 2 weeks and 12 weeks of the dietary intervention. To allow a screen-fail rate and dropout rate, which we predicted would reach >20% based on diet study dropout rates in the literature, 35 participants were recruited.

Statistical Analysis

Thirty-five subjects were enrolled in the study, and 7 subjects dropped out. They were excluded from the analysis; therefore, the results are based on $n = 28$ subjects. Areas under the curve for appetite scores over time were calculated using the trapezoidal rule. The CGM data were calculated for each participant using GNU Octave (version 4.4) software. For time series, a one-way ANOVA was performed to analyze circadian patterns, and a *t* test post hoc analysis was used for comparison between the 6Mdiet and 3Mdiet groups at each time point. In addition, a multivariate ANOVA for repeated measurements was performed assessing between- and within-subject effects for diet and time. Further analysis of circadian rhythmicity was performed using Circwave software (version 1.4) (Circadian Rhythm Laboratory, University of Groningen, Groningen, Holland). The results are expressed as mean ± SEM. A *P* value ≤0.05 was considered statistically significant. Statistical analysis was performed with JMP software (version 14; SAS Institute Inc., Cary, NC).

RESULTS

Participants

Forty-four patients with type 2 diabetes treated with insulin met the inclusion criteria. Nine individuals were excluded: three did not respond after recruiting, three could not commit to the nightly blood sampling, and three were unable to attend all visits to the research center. Thirty-five individuals were randomized and allocated to either the 3Mdiet ($n = 18$) or 6Mdiet group ($n = 17$). Immediately after randomization and before dietary intervention commenced, seven participants dropped out: five patients were unable to follow meal timing and dietary instructions (three patients from 3Mdiet and two patients from 6Mdiet), one patient from the 3Mdiet was excluded because of a new diagnosis of malignancy, and one from the 6Mdiet had health issues requiring another medical follow-up. The data of the 28 patients, which completed the study, were analyzed. Baseline characteristics of the participants, including anthropometric parameters and medical history of diabetes, did not significantly differ between the groups (Table 1). Twenty-seven out of 28 patients were treated with antihypertensive and lipid-lowering drugs.

Body Weight and BMI

After 2 weeks of diet intervention, the 3Mdiet led to a significant weight loss (1.5 ± 0.3 kg) ($P < 0.01$) compared with a nonsignificant weight loss (0.3 ± 0.3 kg) ($P = 0.27$) on the 6Mdiet (Fig. 2*A*). This change led to a significant difference ($P < 0.01$) of 1.2 kg between the groups. After 12 weeks, the 3Mdiet led to a greater weight loss (5.4 ± 0.9 kg, 5.9%) ($P < 0.0001$) compared with a nonsignificant weight gain (0.3 ± 0.3 kg) ($P = 0.27$) in the 6Mdiet group. Accordingly, compared with the 6Mdiet, the BMI was significantly lower in the 3Mdiet group ($P < 0.0001$). Notably, at the end of the study, 12 participants (85.7%) on the 3Mdiet lost ≥2 kg, compared with only two participants (14.3%) on the 6Mdiet.

HbA_{1c}

Over 12 weeks, the 3Mdiet led to a 12 mmol/mol (1.2%) decrease in HbA_{1c}

(from 65.6 ± 10.9 mmol/mol [8.2 ± 0.3%] to 53 ± 9 mmol/mol [7 ± 0.2%]) ($P < 0.0001$) compared with a nonsignificant decrease on the 6Mdiet ($P = 0.5$). This reduction led to a statistically significant difference between the groups ($P = 0.04$) favoring the 3Mdiet over the 6Mdiet intervention (Fig. 2*B*).

Fasting, Overall, and Nocturnal Glucose Levels

After 2 weeks of diet intervention, fasting glucose decreased significantly only in the 3Mdiet group ($P = 0.019$) (Fig. 2*C*). After 12 weeks, fasting glucose was significantly reduced in both groups, but with a greater reduction in the 3Mdiet group (from 165 ± 7 mg/dL [9.2 ± 0.3 mmol/L] to 110 ± 6 mg/dL [6.1 ± 0.3 mmol/L]) compared with the 6Mdiet group (from 164 ± 8 mg/dL [9.2 ± 0.4 mmol/L] to 141 ± 8 mg/dL [7.8 ± 0.4 mmol/L]) ($P = 0.005$) (Fig. 2*C*). The 3Mdiet led to a significant reduction of the daily 24-h mean glucose levels (29.4 ± 11.6 mg/dL [1.6 ± 0.6 mmol/L] and 40 ± 10 mg/dL [2.2 ± 0.6 mmol/L]) ($P < 0.05$) after 2 and 12 weeks, respectively. There was no significant change in daily 24-h mean glucose levels in the 6Mdiet group ($P > 0.05$) (Fig. 2*D*). As a result, at the end of the study, mean daily 24-h glucose was significantly lower on the 3Mdiet compared with the 6Mdiet (129 ± 3 mg/dL [7.2 ± 0.1 mmol/L] vs. 156 ± 11 mg/dL [8.6 ± 0.6 mmol/L]) ($P = 0.03$) (Fig. 2*D*). Similarly, after 12 weeks, a significant difference in the nocturnal (0000–0600 h) mean glucose levels was 108.8 ± 5 mg/dL (6.1 ± 0.3 mmol/L) on the 3Mdiet vs. 141.3 ± 13 mg/dL (7.8 ± 0.7 mmol/L) on the 6Mdiet ($P = 0.03$) (Fig. 2*E*).

CGM assessment in the 3Mdiet group showed a significant increase in the time spent in normoglycemia, from 14 h 14 min (59%) at baseline to 18 h 4 min (75%) after 2 weeks ($P < 0.05$), which further increased to 20 h 10 min (83%) ($P < 0.01$) after 12 weeks. In contrast, the 6Mdiet did not change the time spent in normoglycemia throughout the study (Fig. 3). Similarly, the nighttime spent in normoglycemia was significantly increased in the 3Mdiet group ($P < 0.05$), from 4 h 18 min (72%) at baseline to 5 h 14 min (87%) after 12 weeks compared with a nonsignificant change on the 6Mdiet (Fig. 3).

The daily time spent in hyperglycemia (>180 mg/dL and >10 mmol/L) was significantly reduced ($P < 0.05$) in the 3Mdiet group, from 8 h 59 min (37%) at baseline to 4 h 41 min (20%) after 2 weeks, which further decreased to 3 h 3 min (13%) after 12 weeks ($P < 0.01$) (Fig. 3). In contrast, the 6Mdiet group, remained without a change (Fig. 3). The nocturnal time (0000–0600 h) spent on hyperglycemia was also significantly reduced ($P < 0.05$) only in the 3Mdiet group, from 1 h 18 min (22%) at baseline to 20 min (6%) after 12 weeks compared with no change on the 6Mdiet ($P = 0.06$) (Fig. 3). The daily and nocturnal time spent in hypoglycemia (<70 mg/dL and 3.9 mmol/L) was low (<5%) at baseline and did not change in both groups throughout the study (Fig. 3). Neither minor nor major hypoglycemic episodes were recorded in the groups throughout the study. Therefore, it is noteworthy that despite the significant improvement in the overall and nocturnal glycemia in the 3Mdiet group, it was not associated with any increase in the number of hypoglycemic events (Fig. 3).

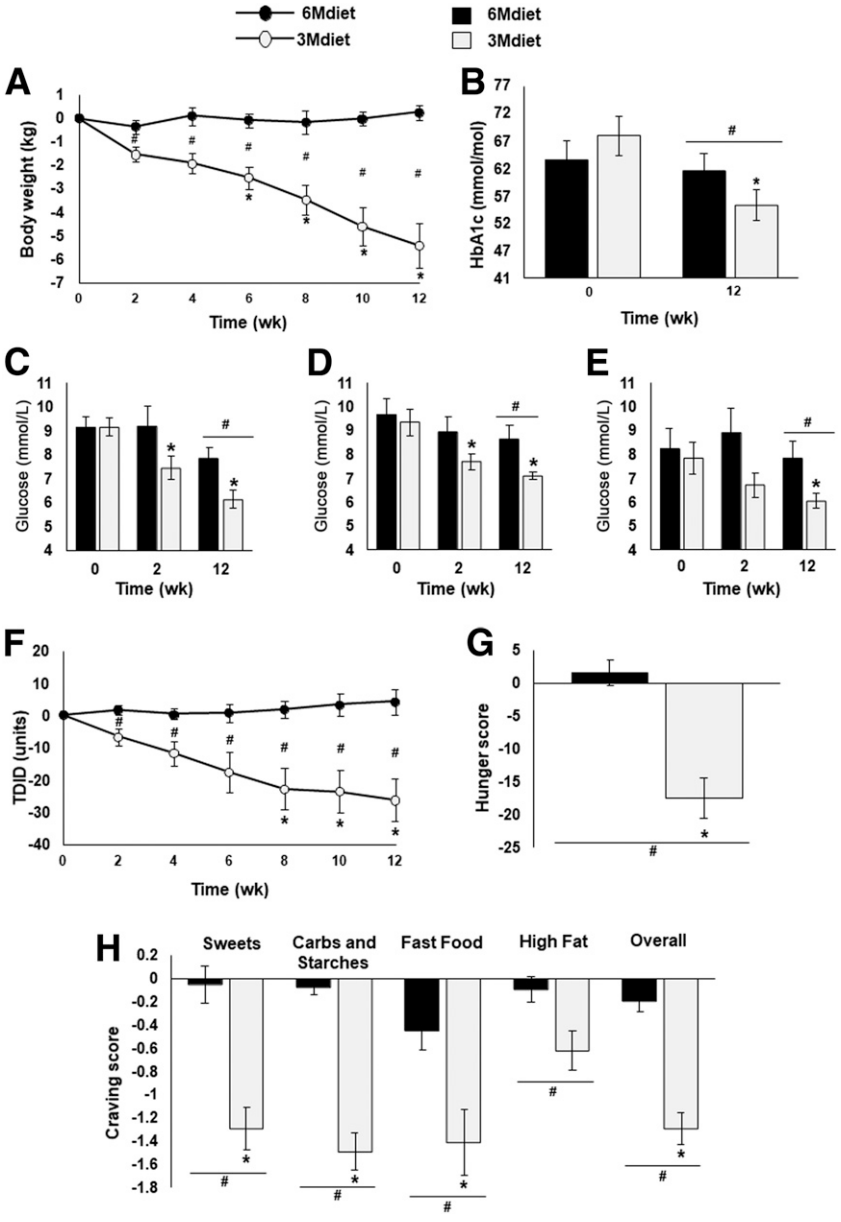

Figure 2—Body weight, HbA_{1c}, glucose levels, TDID, hunger, and cravings at baseline, 2 weeks (wk), and 12 weeks of 3Mdiet and 6Mdiet. *A*: Weight loss. *B*: HbA_{1c}. *C*: Fasting glucose. *D*: Twenty-four–hour mean glucose. *E*: Nocturnal (0000–0600 h) mean glucose. *F*: TDID. *G*: Hunger scores. *H*: Mean daily craving scores. Values are mean ± SE. *Significant difference within groups compared with baseline, $P < 0.05$; #significant difference between groups, $P < 0.05$.

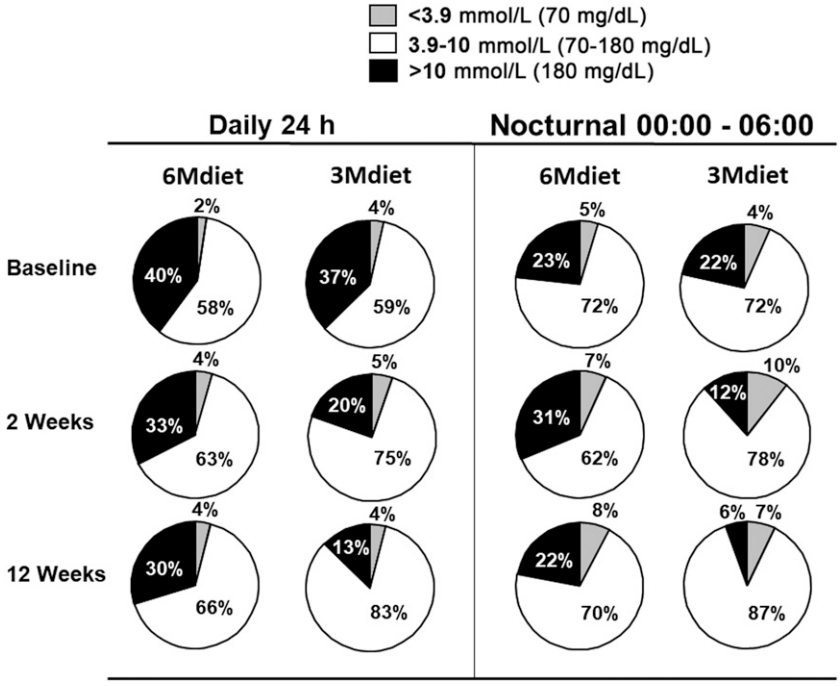

Figure 3—Percentage of daily (24 h) and nocturnal (0000–0600 h) glucose levels spent in range at baseline, 2 weeks, and 12 weeks of 3Mdiet and 6Mdiet.

TDID

The TDID in the 3Mdiet group was reduced significantly, by 7 ± 3 units (from 60 ± 8 at baseline to 53 ± 6.5 units) ($P < 0.05$) after 2 weeks and by 26 ± 7 units (from 60 ± 8 at baseline to 34 ± 7 units) ($P < 0.05$) after 12 weeks. This reduction consisted of 21 ± 5 units of long-acting insulin and 5 ± 4 units of short-acting insulin. In contrast, in the 6Mdiet group, after 12 weeks, there was a nonsignificant increase of 4 ± 3.7 units. This increase resulted mostly from an increase of 8 ± 5 units of the short-acting insulin and a reduction of 4 ± 3 units of the long-acting insulin. As a result, after 12 weeks, the 3Mdiet group was treated with significantly lower TDID of 34 ± 7 units vs. 76 ± 15 units in the 6Mdiet group ($P = 0.001$) (Fig. 2*F*). In the 3Mdiet group, after 12 weeks, a strong positive correlation ($R^2 = 0.652$; $P = 0.002$) was observed between mean daily glucose levels and TDID, suggesting that the daily mean glucose was reduced despite the reduction in TDID. It is noteworthy that throughout the study, neither of the groups showed correlation between body weight and TDID, suggesting that the significant reduction in TDID in the 3Mdiet group was independent of weight loss.

Appetite and Cravings

After 12 weeks of diet intervention, there was a significant decrease in the hunger of the 3Mdiet group ($P < 0.01$), but no change in the 6Mdiet group (Fig. 2*G*). Likewise, the desire for sweets in the afternoon segment (1700–1900) was significantly reduced in the 3Mdiet versus the 6Mdiet group ($P < 0.0001$). After 12 weeks, the 3Mdiet group had a significant reduction in overall daily cravings and the craving sensation for sweets, carbohydrates and starchy food, fast food, and high-fat food ($P < 0.0001$), compared with no significant changes in the 6Mdiet group for any of the food categories (Fig. 2*H*).

Clock Gene Expression

At baseline, participants with type 2 diabetes had reduced diurnal amplitudes in core clock gene expression assessed in WBCs. *PER2* and *RORα* showed no oscillation ($P > 0.05$), while *CRY1* and *BMAL1* presented a rhythmic oscillation at baseline ($P < 0.05$) (Fig. 4). In both diet groups, *BMAL1* expression became oscillatory after 2 weeks ($P < 0.05$) with a 10.5-fold higher amplitude in the 3Mdiet group compared with the 6Mdiet group. *CRY1* presented a rhythmic oscillation throughout the study in the 3Mdiet group ($P < 0.01$), while its expression was dampened in the 6Mdiet group after 12 weeks ($P > 0.05$). After 2 weeks, *PER2* became oscillatory in the 6Mdiet group ($P < 0.01$), but was arrhythmic again at 12 weeks ($P > 0.05$). In the 3Mdiet group, *PER2* expression became oscillatory after 12 weeks ($P < 0.0001$). *RORα* became oscillatory in both diet groups after 2 weeks ($P < 0.05$), but became arrhythmic again after 12 weeks on the 6Mdiet ($P > 0.05$). In the 3Mdiet group, *RORα* continued to oscillate after 12 weeks, with a 5.5-fold higher amplitude compared with baseline (Fig. 4). *PER1* and *REV-ERBα* did not show any change between the 3Mdiet and 6Mdiet groups after 12 weeks of diet intervention (data not shown). The 3Mdiet led to a significant increase in the relative levels of *RORα* and *SIRT1* ($P < 0.01$) after 12 weeks (Fig. 4).

CONCLUSIONS

In this study, we show that a 3Mdiet, with most of the macronutrients shifted to the early hours of the day, improved HbA_{1c} by 12 mmol/mol (1.2%) and reduced body weight by 5 kg in association with reduced appetite, overall and nocturnal glucose excursions, and substantial reduction in TDID in insulin-treated patients with type 2 diabetes. The results are strikingly different from those of the 6Mdiet, with macronutrients uniformly distributed throughout the day. Concomitantly, the 3Mdiet led to a significant upregulation and oscillation of clock gene expression known to be involved in adipogenesis, appetite, and glucose homeostasis.

The different outcomes of the two diets confirm previous studies in obese patients and patients with type 2 diabetes, showing that fewer meals, mainly with increased carbohydrate intake at breakfast and reduced carbohydrate intake at dinner, improve overall glycemia, HbA_{1c}, weight loss, and lipid levels and also reduce hunger and cravings, compared with the reverse schedule (21,22,24,26,31) or to six meals evenly distributed across the day (25). The weight loss of 5.9% observed in the 3Mdiet is consistent with previous studies in type 2 diabetes (25,32) and has been associated with improved insulin sensitivity, β-cell function, and reduced risk factors for cardiometabolic disease (35). Nevertheless, it was also shown that this reduction in body weight might not

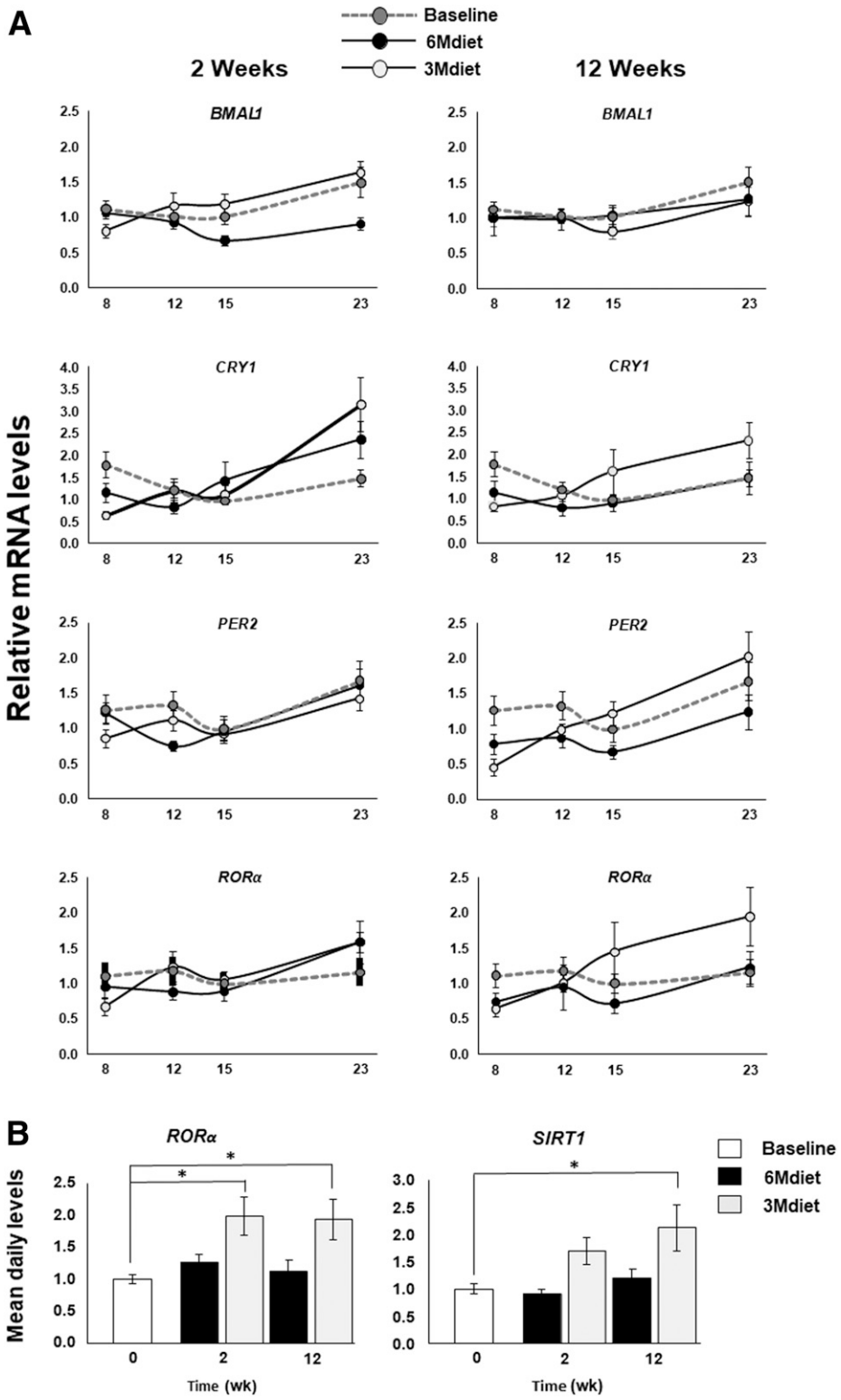

Figure 4—Circadian gene expression in WBCs at 2 and 12 weeks (wk) compared with baseline. *A*: Clock gene expression. *B*: *RORα* and *SIRT1* mean daily levels. Data presented as mean ± SE. *Significant differences from baseline, $P < 0.05$.

be enough to influence the standard measures of overall glycemic control (35). Indeed, in this study, the improvement of glycemic parameters was independent of weight loss. A circadian pattern of diet-induced thermogenesis peaking after high-energy breakfast versus evening meals may explain in part why the 3Mdiet led to more efficient weight loss (18,20,27,36).

The 12 mmol/mol (1.2%) reduction in HbA_{1c} levels found in the participants assigned to the 3Mdiet is supported by previous studies with a similar diet schedule in patients with type 2 diabetes

treated with oral antidiabetic medications (26,32). Moreover, in large epidemiological and clinical studies, it was shown that the risk for diabetes, obesity, postprandial hyperglycemia, and HbA_{1c} is higher among those who eat frequent small meals along the day compared with three daily meals (10–12). It is noteworthy that the reduction in HbA_{1c} in the 3Mdiet group is comparable to the decrease obtained by the addition of GLP-1 receptor agonists (0.65–1.7%) (6,37) or SGLT2 inhibitors (0.5–1.2%) (38) in patients with type 2 diabetes treated with insulin. In light of the HbA_{1c} results, the 24% and 48% reduction in mean daily glucose and the time spent on hyperglycemia, respectively, are comparable with 17–42% reduction of the time spent in hyperglycemia with the use of GLP-1 agonists together with insulin therapy (37). Moreover, the significant reduction in fasting glucose and daily glucose excursions in the 3Mdiet achieved using the Treat to Target protocol (7) led to a 43% reduction in TDID after 12 weeks. This reduction is comparable with the decrease in insulin dose achieved by the addition of liraglutide or SGLT2 inhibitors to basal insulin therapy (37,38). These findings suggest that the 3Mdiet intervention might be equal or even more efficient than pharmacological agents for the reduction of insulin dose requirements.

The 3Mdiet significantly reduced mean daily hunger scores, desire for sweets in the afternoon and evening, and overall cravings. Similar results were achieved in previous studies (22,24,25,32). Hunger and desire for sweets follow a circadian rhythm, with lower rating for hunger and desire for sweets at 0800 and the highest at 2000 h (39). Cravings were shown to be at the highest levels in the late afternoon and early evening, between 1600 and 1900 h (40,41). As proposed predictors of poor adherence to the diet and weight regain include increased subjective appetite scores, especially increased hunger, desire for sweets, and cravings (40,41), the significant reduction in hunger, cravings, and desire for sweets in the afternoon places the 3Mdiet as a preferred diet intervention.

We found disrupted clock gene expression at baseline in both groups. These results are supported by previous findings in patients with type 2 diabetes (30,42,43). Moreover, lower transcripts of *BMAL1* and *CRY2* were inversely correlated with HbA_{1c} levels (43). Disrupted clock genes, such as *BMAL1*, *PER2*, and *CRY1*, were also found in subcutaneous adipose tissue in individuals with type 2 diabetes compared with healthy individuals (42). It has been reported that the disruption or asynchrony of clock gene expression is associated with deficient β-cell responsiveness, secretion, proliferation, and increased apoptosis (16,19), deficient insulin-mediated muscular glucose uptake (15), excessive hepatic glucose production (19), and deficient circadian regulation of lipid mobilization (nocturnal lipolysis) (42). Thus, in light of the literature data, we speculate that the disrupted clock gene expression could be the underlying mechanism for the uncontrolled daily and nocturnal hyperglycemia found at baseline. The disruption of clock gene expression could stem from misalignment or asynchrony between meal timing and the rhythm imposed by the internal circadian clock (i.e., by overeating at late hours of the day) (44), skipping breakfast (29,30), or snacking all day (28). Indeed, in this study, the 6Mdiet did not lead to a significant improvement or amplification of the disrupted clock genes. However, in contrast, the 3Mdiet led to a significant upregulation of *BMAL1*, *CRY1*, *PER2*, and *RORα* oscillation and amplitude and to increased *RORα* and *SIRT1* levels.

The upregulation of clock gene expression in the 3Mdiet may provide a molecular explanation for the attenuated mean daily glucose and the reduced time spent in hyperglycemia with substantially lower daily insulin dose requirements. The upregulation of *BMAL1*, as observed in the 3Mdiet, has been found to be necessary for appropriate glucose-stimulated β-cell insulin secretion (16) and improvement of insulin-stimulated glucose uptake by skeletal muscle, due to enhanced expression and plasma membrane translocation of GLUT4 and increased expression and enzymatic activity of key metabolic enzymes essential for glucose metabolism (15). In addition, BMAL1 activity has been associated with β-cell compensatory expansion, replicative capacity, and survival in response to the progressive insulin resistance in type 2 diabetes (13,45). Thus, *BMAL1* upregulation in the 3Mdiet may be one of the factors potentiating β-cell replication and survival, improving their reserve and capacity for glucose-stimulated insulin secretion, thereby decreasing the needs for exogenous insulin therapy. The 26-unit reduction in TDID after 12 weeks on the 3Mdiet could be attributed to the de novo induction of β-cell secretory potential.

BMAL1 and *CRY2* have also been shown to influence the enzymatic determinants of hepatic glucose output by enhancing glycogen storage and suppressing glucagon-stimulated hepatic glucose production (19,46). These findings together with the increased expression of *BMAL1* could explain the reduction in fasting and postprandial glucose excursions evaluated by the CGM in the 3Mdiet group. Decreased glucotoxicity, because of the reduction in daily and nocturnal hyperglycemia in the 3Mdiet, may also contribute to the reversal of β-cell dysfunction in this group.

Importantly, the 3Mdiet also led to a significant amplification of *RORα* oscillatory expression and to a substantial increase in its relative levels, while no change was observed in the 6Mdiet. *RORα* was found to positively regulate insulin secretion by stimulating the expression of one of the insulin gene transcription factors (47). Moreover, in vivo analyses showed that insulin transcription is enhanced by a synthetic RORα agonist (47). Insulin sensitivity is also influenced by both nutrient state and the clock through SIRT1, as mice on a high-fat diet display decreased SIRT1 levels and impaired insulin sensitivity (48). This suggests that at least partially, the improvement of overall glycemia in the 3Mdiet could be attributed to SIRT1 upregulation.

In type 2 diabetes, the excessive nocturnal hepatic glucose production is attributed to glycogenolysis in the first part of the night (0000–0400 h) and to gluconeogenesis from 0400 to 0700 h. Therefore, fasting glucose reflects mostly hepatic gluconeogenesis (49). Because the 3Mdiet led to a significant reduction in fasting glucose and to diminished glucose levels during both nocturnal segments, it is highly suggestive that both hepatic processes were significantly improved because of the 3Mdiet. *CRY1* and

PER2, which were significantly upregulated in the 3Mdiet, were shown by others to coordinate circadian control over hepatic glucose production through posttranslational regulation of cAMP signaling (46). *CRY1* expression is elevated during the night–day transition, when it inhibits the activation of key gluconeogenic enzymes through direct binding and inhibition of the hepatic glucagon receptor, resulting in attenuated gluconeogenesis and lower fasting and overall glycemia in diabetic mice (19,46). These findings, together with the increased expression of *CRY1* and *PER2* in the 3Mdiet, could explain the significant improvement of nocturnal and fasting glucose levels.

The reduction of hepatic glucose output during the night, without causing hypoglycemia, is one of the most challenging targets in the treatment of type 2 diabetes; therefore, the decrease in overnight glucose, without an increase of hypoglycemic events, is an important advantage of the 3Mdiet. In addition, as exogenous insulin predisposes to weight gain (8) and is associated with increased risk of cardiovascular events and all-cause mortality in a dose-dependent fashion (2), the improvement of glycemic control with significant reduction of TDID in the 3Mdiet is of utmost importance.

One of the limitations of the study includes the analyses of the circadian clock in WBCs. Although the SCN clock synchronizes peripheral clocks, there may be slight differences in the phase of their expression. Thus, the circadian clock in WBCs may slightly differ from other metabolic tissues, such as the liver, muscle, or adipose tissue. Therefore, our results should be corroborated by biopsies taken from metabolic tissues. Another limitation is the fact that sleep was not monitored, as sleep is controlled by the circadian clock, and a change in sleep could alter clock expression.

In conclusion, our results show that, as opposed to the 6Mdiet, the 3Mdiet is an efficient therapeutic means for individuals with type 2 diabetes treated with insulin, as it leads to weight loss, a significant reduction in HbA_{1c}, appetite, overall and nocturnal glycemia, and a substantial decrease of the daily insulin dose. Upregulation of clock genes seen in this diet intervention could contribute to improved glucose metabolism.

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